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(54) Title: ENZYMATIC SYNTHESIS OF N^a-ACYL-L-ARGININE ESTERS

(57) **Abstract:** The invention relates to a process for preparing a Na-acyl-L-arginine ester, derived from fatty acids and esterified dibasic amino acids, according to the following formula (I), where: X- is Br-, Cl-, or HSO₄- R1: is linear alkyl chain from an saturated fatty acid, or hydroxy-acid from 8 to 14 atoms of carbon bonded to the α - amino acid group through amidic bond. R2: is a linear or branched alkyl chain from 1 to 18 carbon atoms or aromatic. R3: is formula (II), where n can be from 0 to 4, from the appropriate organic acid and alcohol. The process is catalyzed by a hydrolase, more in particular a protease, the protease papain from *Carica papaya* being highly suitable. In order for the esterification reaction to run as wanted, the process is performed in a low-water-content organic medium. When the highly suitable papain from *Carica papaya* is used, a water activity of between 0,03 and 0,5 is selected.

ENZYMATIC SYNTHESIS OF N^α-ACYL-L-ARGININE ESTERS

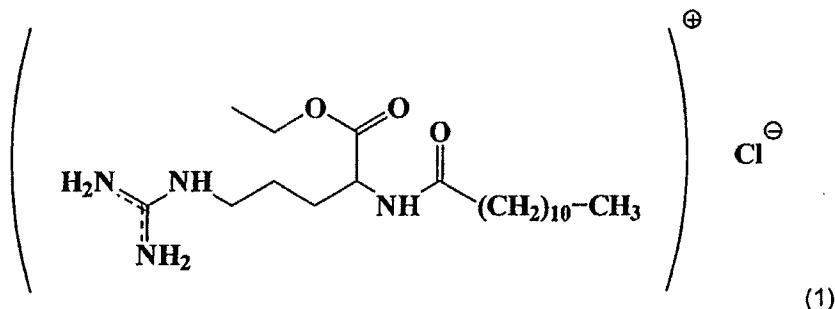
5 DESCRIPTION

This invention relates to a process for preparing N^α-acyl-L-arginine esters with protective activity against microorganisms.

Many antimicrobials are known to protect against specific and general bacteria. But, most of them display incompatibilities with the human skin and the mouth cavity mucous membranes, such as 10 irritations and allergies, and are toxic to human beings as well.

On the other hand, it has been demonstrated that esters derived from lauric acid and L-arginine are biologically active substances, in particular, the ethyl ester of the laurylamide of the L-arginine monohydrochloride, hereafter referred to as LAE. LAE has the chemical structure of formula (1).

15



20

Biological studies carried out at different research centres showed LAE to act mainly over the external and cytoplasmatic membrane of the microorganisms and, also, into the cytoplasmatic medium, preventing their proliferation. Its action depends on the kind of microorganism and on the exposure time.

Besides, its metabolism in rats has been studied showing a fast absorption and metabolism into naturally-occurring amino acids and the fatty acid lauric acid, which are eventually excreted as carbon dioxide and urea. Toxicological studies have demonstrated that LAE is completely harmless to animals and humans.

25

These facts make LAE and related compounds very interesting as preservatives for food and cosmetic applications.

The preparation of these products by traditional chemical methods has been described in patent applications ES-515643, PCT/ES95/00027 and PCT/EP00/05072

It was the object of the present invention to provide a new process to obtain such kind of compounds which is more efficient and selective than the traditional chemical methods, and therefore guarantees final products free of by-products and easy to isolate.

The existence of reversible metabolic degradation pathways for this kind of compounds 5 represents an almost natural way to achieve LAE. Accordingly the process of the invention provides a process by enzymatic reversed reactions in organic solvents to obtain LAE and similar compounds from its corresponding readily available organic acid.

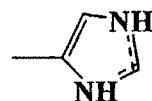
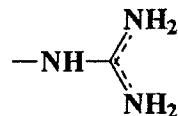
The synthesis of the invention relates to obtain N^{α} -acyl-L-arginine esters according to the 10 formula (2), where:

X^- is Br^- , Cl^- , or HSO_4^-

R_1 is a linear alkyl chain from a saturated fatty acid or hydroxy-acid from 8 to 14 atoms of carbon bonded to the α - amino acid group through an amide bond.

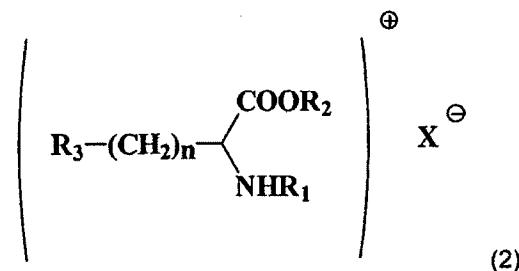
R_2 is a linear or branched alkyl chain from 1 to 18 carbon atoms or an aromatic group.

15 R_3 is:



and n can be from 0 to 4; from the appropriate organic acid of formula (2) where R_2 is -H.

20



The most preferred compound of the above class of compounds is LAE.

The invention is illustrated in the figures 1 to 4:

- Figure 1 - Preparation of LAE, relationship yield-time;
- Figure 2 - Preparation of LAE, relationship yield-time;
- Figure 3 – Device for performing the process of the invention;
- 5 Figure 4 – Preparation of LAE, relationship yield-time.

The biocatalysis using enzymes in reverse hydrolysis reactions to obtain products with important industrial interest is described in many references.

This type of process may be performed in an aqueous medium with or without miscible or
10 immiscible organic solvents, or in a organic solvent, or without any solvent at all. In the latter cases, the reaction naturally needs a minimum content of water for it to take place, as the enzyme otherwise loses its activity under such circumstance. The organic solvent usually contains less than 1% by weight of water, and is referred as low-water-content organic solvent.

Often, the water content is fixed at the beginning of the reaction and no control is performed
15 along the process, however in reactions where water is consumed or produced, the water content becomes a critical value and has to be controlled by addition or elimination of water from the medium using salt hydrates or by other moisturizing or draining physical and/or chemical means.

The water content in an organic medium is frequently measured as water activity. The magnitude of the water activity represents the amount of non-linked water and depends on the water
20 content but also on the nature of the solvent.

The water activity is a thermodynamic parameter defined as the ratio of water vapour's partial pressure above the solution in question to that above pure water of the same temperature and at the same total pressure, when the equilibrium between the liquid and the gas phase has been reached. Then the water activity is essentially equal to the fractional humidity in the gas phase. The water
25 activity of pure water has a value of 1.

Besides the water content, the type of solvent, the pH employed and the temperature may affect kinetics, yield and/or by-products of the process, as all these factors have an influence on enzymatic activity.

As stated before, the catalytic reverse hydrolysis reaction is regularly performed under
30 coinditions of a low amount of water. On the other hand, enzymes are not soluble in organic solvents in spite of the presence of low amounts of water. This has been resolved by the adsorption onto solid supports. Adsorption may be achieved by adding the required amount of adsorbent to the reaction mixture or by providing the enzyme pre-adsorbed onto the support. If the pre-adsorption method is

selected, then the enzyme has to be adsorbed in the correct level of ionisation by using a buffer of the right pH at the adsorption process.

This invention relates to a new method for producing N^{α} -acyl-L-arginine esters with microbiological activity derived from a fatty acid and L-arginine, according to formula (2), using the 5 appropriate organic acid as starting material and enzymes in a low-water-content medium.

The method is based on the enzymatically promoted esterification of a N^{α} -acyl-L-arginine acid, which has a linear alkyl chain from a saturated fatty acid or the hydroxy-acid with 8 to 14 carbon atoms bonded to the α -amino acid group through an amidic bond, with an alcohol consisting of a linear or branched chain from 1 to 18 carbon atoms or a phenylic group. This method has an elevated 10 selectivity, is exempt from by-products, uses mild conditions and involves low toxicity reagents and solvents.

The enzyme may be obtained from a microbial source, or from plant or animal cells or tissues and may be present as a purified or a crude, relatively impure mixture of several different types of enzymes. The enzyme preparation may be in any form such as a free powder, or a lyophilized 15 powder, or covalently linked or adsorbed onto a solid support. The enzyme may be used as a free dispersion in the reaction mixture or onto a solid support, made of materials such as polypropylenes, polyamides, diatomaceous earths, clays, zeolites, activated charcoals, carboxymethyl cellulose, cellulose esters and other substituted celluloses, ion exchange resins, porous glass beads, aluminium oxide, celite or silica gels. The most preferred embodiment for LAE synthesis is using a solid support, 20 and among them, celite. It makes the enzyme easier to remove from the reaction medium by usual methods (e.g. filtration, centrifugation) and to recycle it for a new start of the process.

The enzymes which are suitable for the preparation of the compounds of formula (2) are lipases and esterases, since an ester bond is formed, and peptidases and proteases, as the main substrates are natural amino acid derivatives. Among them, the most appropriate enzymes are 25 proteases, such as bromelaines, clostripain, collagenases, elastases, ficin, kallikreines, metallopeptidases, papain, chymopapain, pepsin, peptidases, proteinases, trypsines, chymotrypsines and carboxypeptidases, but the choice depends on the desired reaction product as the enzyme is chosen by its affinity and specific activity towards both substrates, the organic acid and the alcoholic precursors. So, for the enzymatic synthesis of LAE, clostripain, ficin, kallikreines, papain, 30 chymopapain and trypsines are preferred, most preferably papain from *Carica papaya*.

The enzymes have been adsorbed onto the solid supports referred to previously in the invention according to usual techniques well-known for an expert in the matter, such as lyophilization or humectation, at the appropriate ionization level provided by means of a pH buffer solution. The working pH for papain from *Carica papaya* is from 3 to 10, preferably between 8 and 8,5.

35 As some alcohols are liquid, the reaction may proceed without the further addition of solvents. In all other cases, to disperse enzymatic systems and reagents or to solubilize them, a solvent ought to be used, such as any of sterically hindered alcohols, acetonitrile, cyclic ethers, chlorinated hydrocarbons, ketones, esters, ethers, aromatic hydrocarbons and aliphatic hydrocarbons. In general

all these solvents are suitable, since they dissolve reagents and do not affect the enzymatic activity. The solvent composition is entirely dependent on the product which is desired.

Water or an appropriate buffer solution may also be added, to achieve the optimum water content for the enzymatic activity. So, at a high water content the hydrolysis process is important, but 5 at a very low water content the hydrolysis process does not take place as the enzyme loses its activity. The water activity for papain from *Carica papaya* is between about 0,03 and 0,5, more preferable 0,05 to 0,2, most preferably 0,06 to 0,09.

To increase the yield of the reaction, in some cases, it is necessary to remove the water generated employing a chemical and/or physical draining method, in the form of water exchangers or 10 hydrated salts or chemical dryers or molecular sieves or azeotropic distillation, but at such a rate that the water content in the reaction medium does not drop under the minimum water content for the enzymatic activity. These tools to remove the water may be inside the reaction vessel or in a separate location through which a certain amount of the reaction medium, filtered or not, is recirculated.

The temperature must be set over the freezing point of the solvent, e.g. -50°C for hexane or 15 -45°C for acetonitrile or 11°C for 1,4-dioxane, and below the solvent boiling point, e.g. 81°C for acetonitrile or 101°C for 1,4-dioxane or 76°C for ethanol, more preferably between 15°C and 50°C, most preferably between 25°C and 40°C.

The reaction mixture may also contain further salts, solvents, desiccant agents or enzymes 20 attached to different types of solid supports, which are different from the ones defined in the claims, to achieve the conversion of the mentioned L-arginine derivated acids to the respective esters.

The progress of the reaction is determined by conventional means such as chromatography (thin layer, gas, high performance liquid, and the like) or conductivity measurements. The reaction time may vary from 1h to 7 days, depending on the reaction parameters such as temperature, pH, 25 solvent, the enzyme, solid support, substrates, water activity and draining system. After the complete or desired conversion of the substrates to the final products has been achieved, the enzyme is removed by conventional means such as filtration or centrifugation. Reaction products of high chemical purity are then isolated by conventional means such as simple evaporation of the solvent, crystallization, precipitation, vacuum distillation and/or chromatographic methods.

EXAMPLES

The displayed examples are only a selection, and do not represent a restriction to the conditions, enzymes, solvents or draining systems of the synthetical method in other cases.

- **Example 1:**

5 N^{α} -lauroyl L-arginine hydrochloride (obtained from lauroyl chloride and L-arginine in alkaline medium) is placed in a seal reaction vessel and dissolved in ethanol, containing 0,5% of boric acid/borate 0,1M pH=8,2 buffer, to obtain a N^{α} -lauroyl L-arginine hydrochloride concentration of 32 g/L.

10 A catalyst is prepared which is composed of celite adsorbing 8% of the enzyme papain from *Carica papaya*. The adsorption is performed at pH=8,2 and the preparation comprises 8% by weight of the enzyme relative to the adsorbent and 4% of 1,4-dithio-DL-threitol by weight of adsorbent.

The catalytic system is added to the solution in a ratio of 6/1 referred to the amount of N^{α} -lauroyl L-arginine hydrochloride.

15 The system is purged with argon.

The reaction is carried out at 25°C for 7 days and while smoothly stirring. The process evolution is shown in figure 1.

20 After the completion of the reaction the reaction mixture is filtered to remove the adsorbed enzyme and the final product, LAE, is isolated by conventional means. The process yield is 79% of pure LAE.

- **Example 2:**

The process is carried out under the same conditions as example 1, but using ethanol with 0,6% of buffer solution and at a reaction temperature of 40°C for 36 hours. The process evolution is shown in figure 2.

25 The reaction mixture is filtered to remove the adsorbed enzyme and the final product, LAE, is isolated by conventional means. The process yield is 85% of pure LAE.

- **Example 3:**

This example demonstrates the use of a drying agent in an external device.

30 As figure 3 displays, the initial reaction mixture is placed in a closed vessel (a) under inert atmosphere (b) and equipped with:

- a stirring device (c);

- a jacket to keep the temperature at 35°C by a warming or cooling fluid (d); and
- an outlet filter (e), through which a controlled flow of the filtered reaction mixture is taken by a peristaltic pump (f). This flow is passed through a molecular sieve drying column (g) in such a way that the water activity in the vessel is kept at about 0,06.

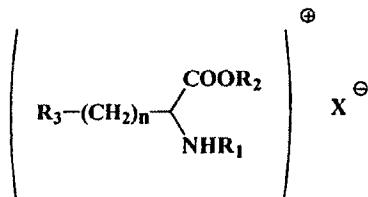
5 The initial reaction mixture has the same composition as example 2. The reaction is carried out for 24 hours at 35°C.

The process evolution is shown in figure 4.

The reaction mixture is filtered to remove the adsorbed enzyme and final product, LAE, is isolated by conventional means. The process yield is 98% of pure LAE.

CLAIMS

1. Process for preparing a N^{α} -acyl-L-arginine ester, derived from fatty acids and esterified dibasic amino acids, according to the following formula:



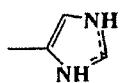
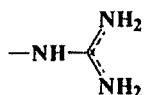
5

where:

 X^{\ominus} is Br^{\ominus} , Cl^{\ominus} , or HSO_4^{\ominus}

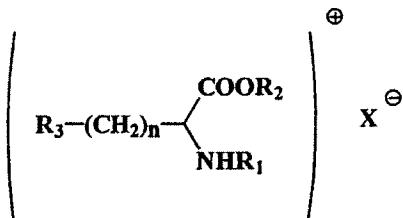
R_1 : is linear alkyl chain from an saturated fatty acid, or hydroxy-acid from 8 to 14 atoms of carbon bonded to the α - amino acid group through amidic bond.

- 10 R_2 : is a linear or branched alkyl chain from 1 to 18 carbon atoms or aromatic.

 R_3 : is:

- 15 where n can be from 0 to 4, from the appropriate organic acid and alcohol, catalyzed by a hydrolase in a low-water-content organic medium.

2. The process as claimed in Claim 1, wherein the starting substrates are an alcohol with linear or branched alkyl chain from 1 to 18 carbon atoms or aromatic; and a N^{α} -acyl-L-arginine acid, as cationic salt or acid salt, according to the following formula:



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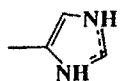
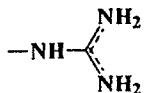
where:

X is Br^- , Cl^- , or HSO_4^-

R_1 : is linear alkyl chain from an saturated fatty acid, or hydroxy-acid from 8 to 14 atoms of carbon bonded to the α - amino acid group through amide bond.

R_2 : is H or an organic or inorganic cation

5 R_3 : is:

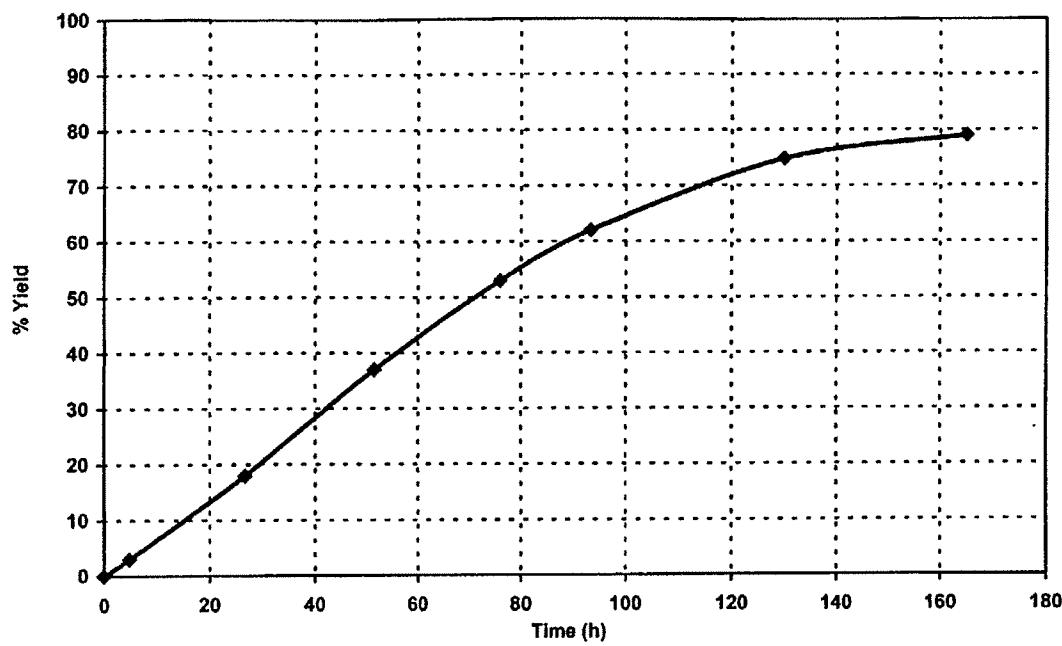


where n can be from 0 to 4.

- 10 3. The process ester as claimed in Claim 1, wherein the N^α -acyl-L-arginine ester is the ethyl ester of the lauryl amide of L-arginine (LAE).
4. The process as claimed in Claim 2, wherein the starting N^α -acyl-L-arginine acid is the N^α -lauryl amide of L-arginine.
5. The process as claimed in any of Claims 1 to 4, wherein said hydrolase is a protease.
- 15 6. The process as claimed in Claim 5, wherein said protease is papain from *Carica papaya*.
7. The process as claimed in any of Claims 1 to 6, wherein the enzyme is adsorbed onto a solid support selected from the group consisting of polypropylenes, polyamides, diatomaceous earths, clays, zeolites, activated charcoals, carboxymethyl cellulose, cellulose esters and other substituted celluloses, ion exchange resins, insoluble polysaccharides, porous glass beads, 20 aluminium oxide, celite and silica gels.
8. The process as claimed in Claim 7, wherein the enzyme adsorption onto the solid support is carried out by lyophilisation or humectation of a mixture of the solid support and a dispersion of the enzymatic catalyst in the appropriate buffer solution.
9. The process as claimed in any of Claims 1 to 8, wherein the reaction solvent is selected from the 25 group consisting of sterically hindered alcohols, acetonitrile, cyclic ethers, chlorinated hydrocarbons, ketones, esters, ethers, aromatic hydrocarbons, aliphatic hydrocarbons and mixtures of them.
10. The process as claimed in any of Claims 1 to 9, wherein the reaction is performed at a water activity between 0,02 and 0,1.

11. The process as claimed in any of Claims 1 to 10, wherein the reaction is performed at a temperature between 20° C and 45° C.
12. The process as claimed in any of Claims 1 to 11, wherein the reaction is performed at a pH between 3 and 10.
- 5 13. The process as claimed in any of Claims 1 to 12, wherein the water generated in the reaction mixture is drained by a drying agent or a physical method, placed inside or outside of the reaction vessel.

10



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Figure 1

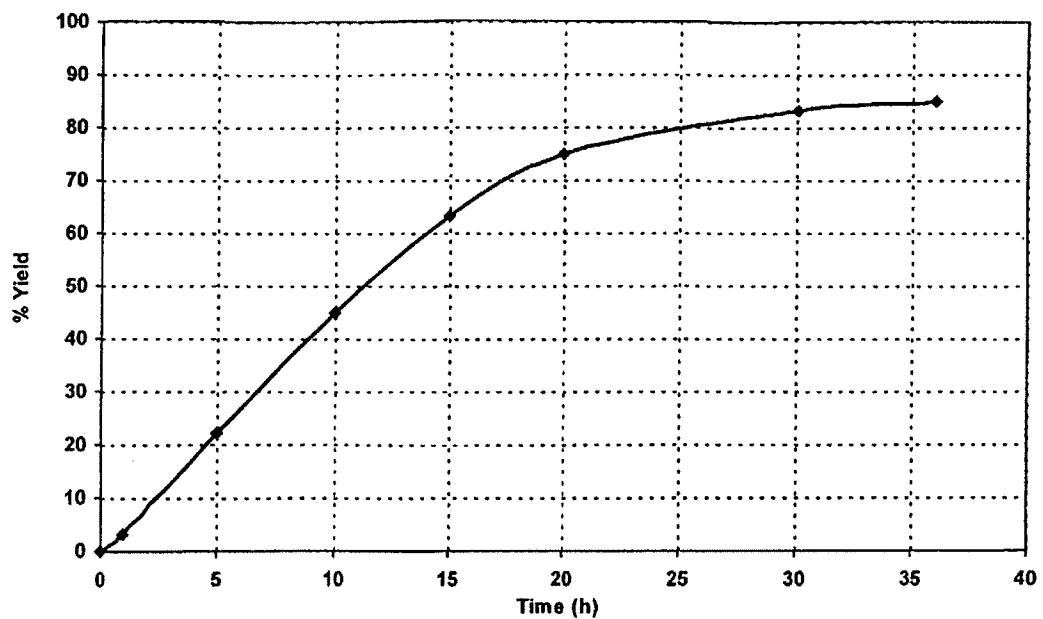
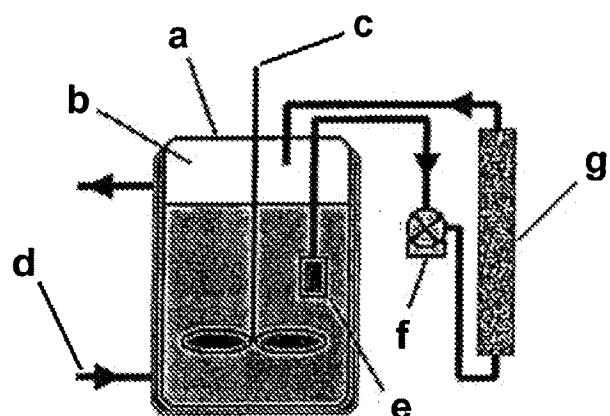


Figure 2



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Figure 3

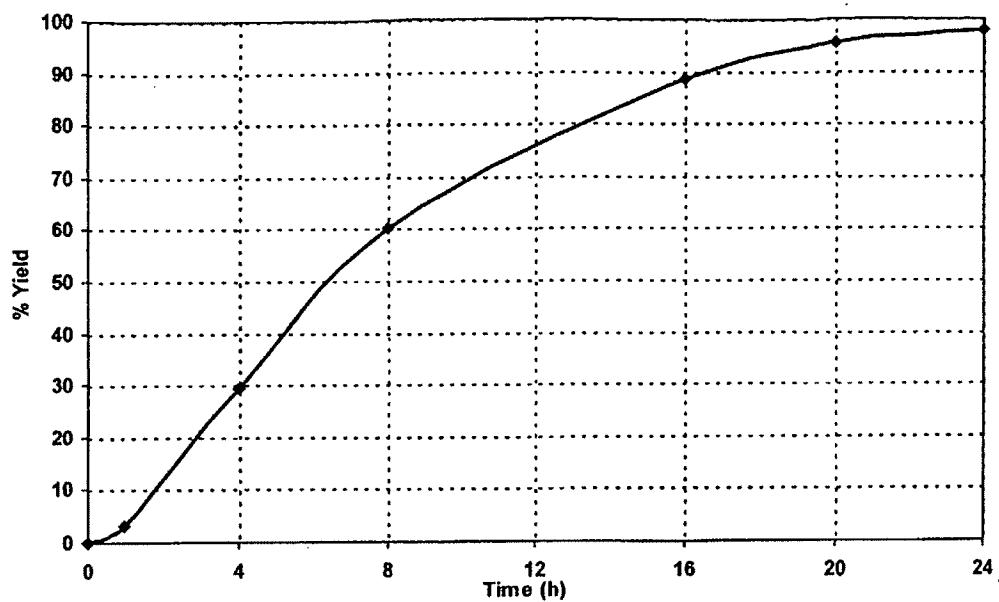


Figure 4

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/01070

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12P13/04 C12P13/08 C12P13/10 C12P13/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C12P C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, EPO-Internal, WPI Data, CHEM ABS Data, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRAUN K ET AL: "Papain-catalyzed esterification of N-alpha-protected amino acids and dipeptides with ethanol in different organic systems." PHARMAZIE, vol. 52, no. 3, 1997, pages 203-206, XP001093713 ISSN: 0031-7144 abstract; figures 1-4 page 203, column 2, line 39 -page 204, column 1, line 2	1-13
A	WO 97 30964 A (YISSUM RES DEV CO ;EYAL AHARON M (IL); SAXE BERNHARD D (US)) 28 August 1997 (1997-08-28) page 11, line 24 - line 25; claims 1,21,22	1-13 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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Date of the actual completion of the International search

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/01070

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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